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(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
10 January 2002 (10.01.2002)

PCT

(10) International Publication Number
WO 02/02155 A1

(51) International Patent Classification⁷: **A61L 15/38**

(21) International Application Number: **PCT/EP00/06278**

(22) International Filing Date: **4 July 2000 (04.07.2000)**

(25) Filing Language: **English**

(26) Publication Language: **English**

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(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

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WO 02/02155 A1

(54) Title: **WOUND DRESSING COMPRISING A THERAPEUTICALLY ACTIVE AGENT**

(57) Abstract: The present invention relates to wound dressings comprising an improved therapeutic effect due to the presence of the therapeutically active agent attached thereto. The invention provides a method which allows an optimal correlation between the degree of oxidation of a cellulose-based carrier and the amount of the therapeutically active agent attached thereto. The invention further discloses the use of the enzyme terrilytin in combination with other proteins as therapeutic agents. Furthermore, a method of manufacturing said dressing is disclosed.

containing layer in between. U.S. Pat. No. 5,836,970 teaches the use of a wound dressing comprising a combination of chitosan and alginate in order to achieve a bacteriostatic and hemostatic effect. The wound dressing is provided in the form of a powder, film, gel, foam
5 or mixtures thereof. According to the teaching of U.S. Pat. No. 5,098,417 a wound dressing is disclosed for systematic administration of physiologically or biologically active agent adsorbed thereon by ionic interaction.

RU 2,131,268 discloses a dressing material with curative properties based on medicinal
10 cellulose-derived gauze. Trypsin alone or in combination with insulin is chemically attached to dialdehyde groups which have been introduced by oxidation into cellulose used as carrier material. It was shown that, in principle, dressings with trypsin attached thereon could be effective in the treatment of pyo-necrotic wounds of most etiologies. However, under these conditions trypsin has only a reduced therapeutic effect. First, the quantity of attached
15 trypsin is very low (0.25 mg per gram of the carrier) and secondly, the degree of oxidation of cellulosic moities is very high (2 %). Most of the enzyme turns out to be covalently bound to the carrier in a way which leads to reduced therapeutic efficacy. The 3-D structure of the enzyme is distorted, the conformational flexibility restrained and the relative activity of the chemically bound enzyme diminished when compared to native enzyme. Frequent changing
20 of the dressing is required in order to achieve the therapeutic effect.

RU 2,142,818 describes a so-called Filatov-Ryltsev dressing, a partly oxidized medicinal gauze treated with trypsin alone, or trypsin in combination with lysozyme or insulin. Nevertheless, the therapeutic effect, is reduced due to the low degree of oxidation of
25 cellulosic moities (0.32-0.48%). As a result most of the enzyme attached to the carrier gets rapidly washed into the wound discharge.

Moreover trypsin, a serine protease which is widely used in medicinal treatment, is relatively expensive due to the labor- and time-consuming steps required for its isolation. Since it is
30 usually isolated from the bovine pancreas, the application of trypsin in the field of wound dressings is also hampered with respect to consistent quality of the protease and by the possibility of transmitting diseases.

The invention teaches the attachment of the therapeutically active agent or agents to the carrier in effective quantities, with an attachment mechanism and/or orientation which maintains therapeutic activity. Preferred therapeutic agents are proteinaceous, proteolytic enzymes being particularly preferred agents. The therapeutically active agent could comprise one more proteins or protein derivatives which has been isolated from natural sources and/or from hosts genetically engineered according to recombinant DNA methods known in the art. In particular, insulin or hen egg lysozyme are preferred, especially recombinantly produced versions of these proteins.

The invention provides the use of the proteolytic enzyme terrilytin as therapeutic agent. Terrilytin not only surpasses the properties of trypsin when used for the treatment of pyo-necrotic wounds but is cost-effective as well. The invention further provides the use of terrilytin in combination with at least one additional protein which has been selected in such a way that synergy in their therapeutic effect occurs. A combination of terrilytin, lysozyme and another protein, especially insulin, is preferred.

The invention also provides a method for the preparation of said wound dressings using a carrier in the form of a fabric or cloth. Preferred methods are those wherein the carrier is activated prior to attachment of the therapeutic agent, and those characterized in that the agent attached to said carrier is present in at least three therapeutically active fractions (termed herein the therapeutically active "triple system").

Accordingly, a wound dressing is claimed comprising a therapeutically active agent and a cellulose-based carrier, wherein the therapeutically active agent comprises the enzyme terrilytin and at least one additional protein is attached to said carrier. Moreover, a wound dressing is claimed wherein the wound dressing comprises at least a therapeutically active system comprising three fractions of therapeutically active agent, namely an adsorbed fraction, a "stabilized fraction" and an "immobilized fraction". The wound dressings of this invention and the therapeutically active triple system comprising the therapeutically active agent are claimed for use for the treatment of pyo-necrotic wounds.

Furthermore, a method for manufacturing wound dressings according to the invention is claimed which comprises the steps of activating said carrier by oxidizing an initial medicinal

A method is disclosed for the preparation of said wound dressing comprising an activation step of the cellulose-based carrier followed by the attachment of the therapeutically active agent thereto. Within the context of the invention, "activation" is understood as the chemical or physical modification of the cellulosic portion of the carrier in order to improve the attachment of the therapeutically active agent of the present invention. A representative example for the activation is the oxidation of at least some glucoside units to dialdehyde groups (i.e. dialdehyde cellulose, DAC). Within the scope of the present invention, the term "attached" is understood as the permanent or temporary loading of the therapeutically active agent of the present invention onto the cellulose-based carrier, based on any kind of interaction such as, but is not limited to, ionic interaction, van der Waals-interaction, dipole-dipole interaction, hydrogen bonds and covalent bonds.

The enzyme terrilytin (CAS RN 37338-91-3) has, like trypsin, proteolytic activity but offers several additional advantages. Terrilytin is produced by the fungus *Aspergillus terricola* and was shown to be a complex comprising three proteolytic enzymes. Two of the proteases (proteases I and II) are of the group of serine proteases as judged by their physico-chemical and enzymatic characteristics and might be considered as two sub-types of the same enzyme. Protease III shows characteristics specific for metalloproteases of microbial origin (Seleznova and Bol'shakova, *Prikl. Biokhim. Mikrobiol.* 22: 3-11, 1986). The medicinal use of terrilytin, e.g. its fibrinolytic and anti-inflammatory properties, has been shown (M. D. Mashkovsky, *Therapeutic Remedies*, Vol. 2, pp. 60-61, Meditsina Publishing House, Moscow, 1993). The proteolytic activity of terrilytin is the sum of the activities of all subunits. The term "proteolytic unit" describes quantitatively the ability of proteolytic cleavage of peptide bonds in proteins. For more details see Byelov et al., *Khimiko-farmatsevtichesky Zhurnal* 12: 101-103, 1992.

Terrilytin offers several advantages over trypsin with respect to manufacturing wound dressings for the treatment of pyo-necrotic wounds. Firstly, terrilytin can be obtained on a commercial scale with consistent quality using microbiological techniques such as industrial fermentation. Secondly, enzymes of microbial origin, in contrast to those from animal sources like trypsin, generally tend to have a higher specific activity due to an increased degree of purity of the enzyme in the preparation. Thirdly, in contrast to trypsin, the possibility of transmitting disease is substantially reduced due to the microbiological origin

terrilytin and lysozyme treatment. This results in a further advantage of the present invention: the reduced necessity to exchange the applied wound dressing during a single course of treatment, namely from 3 napkins in the case of wound dressings comprising the cellulose-based carrier with trypsin alone or trypsin in combination with other proteins,
5 down to only 1 napkin when using the carrier terrilytin alone or terrilytin in combination with other proteins.

It is not obvious for people skilled in the field of wound treatment to combine a proteolytic enzyme like terrilytin with a protein like the hormone insulin. Experimentation, however,
10 shows a surprising synergistic effect of this combination by accelerating the cleaning process of a wound as judged from the degradation of debris and healing of trophic ulcers. Wound dressings manufactured according to the invention which carry both terrilytin and insulin attached thereon exert curative effects not only during the first stage of wound healing, but
15 also during the regeneration of lost soft tissue. The terrilytin-insulin wound dressing strongly improves cellular proliferation, granulation growth and epithelisation. A complete cleaning of the wound from debris is observed after two days, versus three days using techniques disclosed in the prior art.

20 This surprising synergism is helpful for the treatment of pyo-necrotic wounds of various etiologies, such as but not limited to proctitis, paraproctitis, otitis, decubitus sores, trophic ulcers, burns, frost bite, gunshot injuries, incised wounds, gangrene and keloid scars. These types of wounds may frequently lead to the amputation of an extremity. The present invention as disclosed herein, however, offers a new approach to their treatment.

25 For the production of the dressing according to the present invention, a cellulose-based carrier is activated by introducing reactive groups, in particular dialdehyde groups, into the cellulosic portion of the carrier to which the therapeutically active agent is subsequently attached. For example, dialdehyde cellulose (DAC) is produced by exposing medicinal
30 gauze to periodic acid. The oxidative conversion of polysaccharides to dialdehyde cellulose using periodic acid as an oxidizing agent is well known in the art, e.g. see U.S. Pat. No. 4,082,743. In a second step, attachment of therapeutically active agent is accomplished by passing the activated carrier through an aqueous solution containing the desired proteins.

or simultaneously with different protein solutions (in the latter case protein solutions are mixed immediately prior to the treatment). Temperatures above approximately 65° C should generally be avoided. The present process is readily carried out at ambient temperature using a buffered aqueous solution of the proteins. The temperature of choice depends, however, on the particular protein or mixture of proteins used. Usually the temperature ranges from approximately -5° C to approximately 30° C. A temperature in the range from approximately 10° C to approximately 25° C is preferred. Each of the protein concentrations can vary in the range from 0.01 mg/ml to approximately 50 mg/ml, preferably in the range from 0.1 mg/ml to approximately 2 mg/ml each.

The present invention provides a method which allows an optimal correlation between the degree of oxidation of a cellulose-based carrier and the amount of the therapeutically active agent attached thereto. According to the invention the chosen oxidation degree of the activated carrier and the amount of the chosen proteins lead to the presence of at least three fractions ("triple system") of the therapeutically active agent attached to the carrier: an adsorbed fraction, a "stabilized fraction" and an "immobilized fraction".

The three fractions can be characterized as follows: The first fraction comprises a therapeutically effective agent attached to the activated cellulose-based carrier by adsorption only. The second fraction, so-called "stabilized fraction", is formed by the activated cellulose-based carrier being degraded by a hydrolytic process during exposure to the microenvironment of a wound. In this context it should be noted that the properties of the cellulose-based carrier are very different to those of the activated cellulose-based carrier (DAC). While the former is a chemically inert and water-insoluble substance, the later is chemically active and can, at least in part, dissolve in water due to hydrolysis. During the exposure of medicinal gauze to periodic acid, dialdehyde moieties are unevenly introduced. Areas with a higher concentration of dialdehyde groups seem to be more prone to hydrolysis. The immobilization of a protein following the method disclosed in this invention does not alter the patterns of hydrolytic degradation. As a result cellulose oligomers with proteins attached thereto are produced. Areas with a lower concentration of dialdehyde groups are more resistant to hydrolysis so that it takes longer until these areas are degraded to cellulose oligomers. This latter fraction is called "immobilized fraction". The kinetics of the

The present invention is further illustrated by the following examples.

Example 1: Production of dialdehyde cellulose

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14 g of periodic acid is added to 2 L of distilled water in a glass vessel. In another glass vessel, 2.5 g of sodium hydroxide is solved in 2 L of distilled water. Both solutions are combined under agitation. A reactor with a capacity of 10 L is charged with 1 kg of medicinal gauze for use as carrier. The latter is exposed to the freshly prepared 4 L of sodium periodate for 15–20 hours at ambient temperature under intermittent agitation. The final product, dialdehyde cellulose (DAC) with a degree of maximal oxidation of $1.0 \% \pm 0.1\%$, is pressed to remove excess liquid, thoroughly washed with 5 x 10 L water and pressed again.

15 Example 2: Immobilization of terrilytin and lysozyme or insulin

In a glass vessel 0.4 g of terrilytin is dissolved in 2 L of phosphate buffer pH 4.4 ± 0.4 . Similarly, 0.4 g of lysozyme or insulin, respectively, is dissolved in 2 L of phosphate buffer pH 4.4 ± 0.4 . The two solutions are mixed immediately prior to the immobilization process resulting in a final concentration of 0.1 g/L or 0.01 wt.-% each. The combined solutions are quickly added to a 10 L reactor which has been pre-loaded with 1 kg of activated cellulose-based carrier prepared as described in Example 1. After 4 hours at 20° Celsius the product is pressed to remove excess liquid until 1.5 wt.-% remaining moisture is achieved. The product is then incubated under refrigeration (4° Celsius) for another 4 hours. The treatment of DAC under the above conditions gives rise to a product in which virtually all of terrilytin and lysozyme or insulin becomes attached to said carrier (0.4 mg each per 1 g of DAC). The product is air dried until $\leq 10 \%$ humidity is reached, cut into napkins and placed into polyethylene bags. Subsequently, the bags are sealed and sterilized with 25 kGy gamma irradiation.

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Claims

1. Wound dressing comprising a therapeutically active agent and a cellulose-based carrier, wherein the therapeutically active agent comprises the enzyme terrilytin or a derivative thereof and at least one additional protein being attached to said carrier.
2. Wound dressing according to claim 1, wherein the additional protein is genetically engineered.
3. Wound dressing according to claim 1 or 2, wherein the additional protein is insulin.
4. Wound dressing according to claim 1 or 2, wherein the additional protein is lysozyme.
5. Wound dressing according to anyone of claims 1-4, wherein the wound dressing comprises at least a therapeutically active system comprising
- (a) an adsorbed fraction,
 - (b) a stabilized fraction, and
 - (c) an immobilized fraction
- of said therapeutically active agent.
6. Wound dressing according to anyone of claims 1-5, wherein the proteins attached to said carrier consist of terrilytin, lysozyme and insulin.
7. Method for manufacturing a wound dressing comprising the steps of
- activating a cellulose-based carrier by oxidizing an initial medicinal gauze
 - preparing separate protein solutions in buffered solutions;
 - combining the prepared protein solutions to form a combined protein solution and adding the combined protein solution to said oxidized medicinal gauze or
 - alternatively treating said oxidized medicinal gauze sequentially with separate protein solutions;

15. The therapeutically active agent to anyone of claims 1-6 for use for the treatment of pyo-necrotic wounds.
- 5 16. Use of the enzyme terrilytin for the manufacture of a medicament for the treatment of pyo-necrotic wounds, wherein the medicament is the wound dressing according to anyone of claims 1-6.
- 10 17. Wound dressing according to anyone of claims 1-6 comprising three fractions of therapeutically active agent.
18. Wound dressing according to claim 17 for the use for the treatment of pyo-necrotic wounds.
- 15 19. Wound dressing characterized in that the wound dressing comprises at least a therapeutically active system comprising
- (a) an adsorbed fraction,
 - (d) a stabilized fraction, and
 - (e) an immobilized fraction
- 20 of said therapeutically active agent.

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- combining the prepared protein solutions to form a combined protein solution and adding the combined protein solution to said oxidized medicinal gauze or
- alternatively treating said oxidized medicinal gauze sequentially with separate protein solutions;
- allowing the protein solution to react for a period of time sufficient to reach a predetermined level of attachment;
- removing the final product from the protein solution, washing and drying it;

characterized in that the step of oxidizing results in a cellulose-based carrier having an oxidation degree of approximately 0.5–1.5 %

and wherein terrilytin and insulin are attached to said activated carrier in equimolar proportions.

8. Method for manufacturing a wound dressing according to claim 7, wherein said step of oxidizing results in a degree of maximal oxidation of approximately 0.9–1.1 %.
9. Method for manufacturing a wound dressing according to claim 7, wherein the preparation of protein solutions comprises forming solutions each with a protein concentration in the range from 0.01 mg/ml to approximately 50 mg/ml each.
10. Method for manufacturing a wound dressing according to any one of claims 7-9, wherein the protein concentration is in the range from 0.1 mg/ml to approximately 2 mg/ml.
11. Method for manufacturing a wound dressing according to any one of claims 7-10, wherein the reaction product comprises 0.4 mg of each protein per gram of cellulose-based carrier which corresponds to
 - 0.1 proteolytic unit for terrilytin/g of cellulose-based carrier;

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61L15/38

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, CHEM ABS Data, EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GB 2 240 040 A (MO MED STOMATOLOG ;VNII TEXTIL GALANTEREY PROMY (SU)) 24 July 1991 (1991-07-24) page 9, line 16 - line 22 page 13, line 1 - line 15; examples --- -/--	1,2,4, 7-10, 14-18

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

2 March 2001

Date of mailing of the international search report

14/03/2001

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>DATABASE CHEMABS 'Online! CHEMICAL ABSTRACTS SERVICE, COLUMBUS, OHIO, US; YUDANOVA, T. N. ET AL: "Properties of enzyme-containing fibrous biomedical materials" retrieved from STN Database accession no. 118:132079 XP002161990 abstract & IZV. VYSSH. UCHEBN. ZAVED., KHIM. KHIM. TEKHNOL. (1992), 35(9), 95-9 ,</p>	1
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Information on patent family members

| trial Application No | |

PCT/EP 00/06278

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